Site-directed mutagenesis of α-tubulin

Reductive methylation studies of the Lys 394 region

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ABSTRACT Previous studies have implicated at least two regions in α-tubulin that are important for the regulation of microtubule assembly. These regions include a cluster of basic residues consisting of Arg 390, His 393, and Lys 394 and the highly acidic carboxyl terminus. Lys 394 is highly reactive to HCHO and $NaCNBH_3$. The reductive methylation of Lys 394 by these reagents is thought to be responsible for the profound inhibitory effects of low concentrations of HCHO on microtubule assembly (cf. Szasz J., M. B. Yaffe, M. Elzinga, G. S. Blank, and H. Sternlicht. 1986. *Biochemistry.* 25:4572–4582). In this study we reexamined the basis for this inhibition. Lys 394 in a human keratinocyte α-tubulin (kα1) was replaced by a glutamic acid residue using site-directed mutagenesis. The mutant K394E was synthesized in vitro using rabbit reticulocyte lysates, and its ability to coassemble with bovine brain microtubule protein (MTP) before and after reaction with HCHO and $NaCNBH_3$ was compared with that of wild-type. No differences in the coassemblies of the unmethylated proteins were detected suggesting that Lys 394 is not essential for microtubule assembly. However, methylated K394E prepared at low HCHO concentrations (<1 mM) incorporated into microtubules to a greater extent (~30–40%) than methylated wild-type. This result is consistent with the hypothesis that methylation of Lys 394 interferes with microtubule assembly. However, the extent of protection afforded by the replacement of Lys 394 with Glu 394 was less than half as large as that predicted from the earlier studies. We tentatively conclude that another residue(s) besides Lys 394 contributes significantly to the assembly-inhibition observed with low concentrations of HCHO. Since this residue(s) is less reactive than Lys 394, it would have to inhibit assembly substoichiometrically when methylated. Potential candidates for this residue include bulk lysyl residue(s), a lysyl residue(s) with intermediate reactivity toward HCHO, and the NH_2-termini. The NH_2-termini are especially attractive candidates since they appear to have a structural role in microtubule assembly.

INTRODUCTION

Microtubules are cylindical structures that participate in a variety of diverse functions in eucaryotic cells including mitosis, morphogenesis, and vesicle transport (1). A molecular understanding of these functions will involve in part detailed knowledge of the properties of the major constituent protein of the microtuble, tubulin, and its two homologous subunits, α- and β-tubulin. These subunits are each ~450 amino acid residues in length (2, 3), display ca. 40% sequence homology and have different functional roles that at present are only poorly understood (4, 5). The COOH-terminal regions of α- and β-tubulin are important for the regulation of microtubule assembly. These highly negatively charged regions, for example, contain binding sites for microtubule associated proteins as well as metal ions, such as Ca^{2+} (6–10). In contrast, the role of the NH_2-terminal regions in assembly and tubulin function is less well understood, although these regions have been implicated in the autoregulation of tubulin synthesis (11, 12).

We previously reported that the α-subunit contains a highly reactive residue, Lys 394, whose methylation renders tubulin assembly incompetent (13, 14). The enhanced reactivity of this residue was attributed to a lowered pK_a, i.e., to an increase in nucleophilicity induced by its location in a positively charged cluster consisting of Lys 394, His 393, and Arg 390 (14, 15). The basis for the assembly inhibition upon methylation of Lys 394 is not understood, although one attractive hypothesis is that methylation perturbs an essential interaction between the positively charged cluster and the highly negatively charged COOH-terminal region (14, 16). Methylation was accomplished with HCHO and $NaCNBH_3$ under conditions that specifically modify α- and ε-amino groups. NH_2-termini in the native protein were modified but showed a reduced reactivity relative to denatured protein, which was interpreted as evidence that these residues are partially buried in the native dimer or in a salt-bridge interaction (17). Lys 394, on the other hand, is ~10-fold more reactive than the bulk lysyl resides and at least 3-fold more reactive than the NH_2-terminal methionine residues. A nearly stoichiometric correlation was observed between the extent of its methylation and the extent of assembly inhibition at low HCHO concentrations (14, 17). Subsequent studies have implicated Lys 394 in the pathophysiology of diabetes (18) and in alcohol-induced hepatic necrosis (19, 20), two disorders that result in impaired microtubule function. However no biological role has been assigned to Lys 394, although another lysyl residue in α-tubulin, i.e., Lys 40 is a specific substrate for an acetyltransferase (21, 22). Acetylation of Lys 40 does not appear to have a significant effect on microtubule assembly (23).

Recently, we carried out a modification study of tubulin using diethyl pyrocarbonate (DEP)[1], an ethoxyfor-

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[1] *Abbreviations used in this article:* DEP, diethyl pyrocarbonate; HPLC, high-performance liquid chromatography; I.S., initial sample; MAPs, microtubule associated proteins; MTP, microtubule protein;

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mylating reagent that preferentially reacts with histidyl residues but also reacts with α- and ε-amino groups (24, 25). Lys 394 modification was not detected with DEP. Instead we reported that ethoxyformylation of the NH_2-terminal methionyl residues appears to induce a potent, possibly substoichiometric inhibition of microtubule assembly. A similar conclusion was reached in a study with levuglandin E_2, a γ-keto aldehyde derived from the prostaglandin endoperoxide prostaglandin H_2 (26). These findings supported the notion that methylation of the NH_2-termini might have been responsible for assembly inhibition by HCHO and raised questions concerning Lys 394 as an essential residue in microtubule assembly. To address these questions, we undertook a site-directed mutagenesis study of the Lys 394 region in α-tubulin. Lys 394 was replaced by a glutamic acid residue. The mutant α-tubulin was expressed in vitro, and its ability to coassemble with exogenous MTP before and after reductive methylation was investigated. These studies suggest that Lys 394 is not an essential residue per se. However, methylation of this residue did inhibit incorporation into the microtubule lattice, although the extent of inhibition is less than half as large as that expected from the earlier studies of Szasz et al. (13) and Sherman et al. (17). We conclude that additional residues, presumably the NH_2-terminal methionines, contribute significantly to the assembly-inhibition observed with low concentrations of HCHO.

METHODS

Materials

Taxol, a gift from the National Cancer Institute and Flow Laboratories, Inc., was prepared as a 4 mM stock in dimethylsulfoxide, aliquoted, and stored at −20°C. [^{35}S]Methionine (>1,000 Ci/mmol) and [^{3}H]-tyrosine (41 Ci/mmol) were purchased from ICN Biomedicals Inc. (Costa Mesa, CA) and New England Nuclear (Boston, MA), respectively. Colony hybridization was performed using oligonucleotides labeled with [^{32}P]ATP purchased from New England Nuclear. DNA sequencing was done with Sequenase from USB Corp. (Cleveland, OH) and α-[^{35}S]dATP (SJ 304, >600 Ci/mmol) purchased from Amersham (Arlington Heights, IL). Restriction and DNA-modifying enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Standard manipulations of DNA were performed as outlined in Maniatis et al. (27) unless indicated otherwise.

Preparation of microtubule protein

Microtubule protein (MTP) was purified from bovine brain by repetitive cycles of assembly/disassembly following a procedure (28) modified from Gaskin et al. (29). Twice-cycled preparations, consisting of ~80% tubulin and ~20% microtubule-associated proteins (MAPs), were stored in PB + 5 M glycerol at −20°C and used within 2 mo. Protein concentrations were determined by the method of Lowry et al. (30) using bovine serum albumin (BSA) as a standard.

P1 and 2, first and second pellet; PAGE, polyacrylamide gel electrophoresis; S1 and 2, first and second supernatant; SDS, sodium dodecyl sulfate.

Construction of α-tubulin mutants

Oligonucleotide-directed mutagenesis of residues 390 and 394 in α-tubulin were performed with the gap-duplex technique of Morinaga et al. (31), using the parent vector pGEM 3α10, a 4.4-kb plasmid containing a full-length human α-tubulin cDNA inserted into the multiple cloning site of pGEM3 (32). To generate point mutations, pGEM3α10 was digested with Hind III and Sph I to create a 1.5-kbp gap encompassing the α-tubulin gene. A second sample of pGEM3α10 was linearized within the β-lactamase gene by Pvu I and the 5′ overhang filled in with T4 DNA polymerase to disrupt ampicillin resistance in the event of religation. Both fragments (0.03 pmol) were purified by agarose gel electrophoresis, mixed, and combined with ~15 pmol of a phosphorylated synthetic oligonucleotide (19 bp) containing the appropriate point mutation. Samples were denatured at 100°C for 4 min and then renatured by gradual cooling (30 min at room temperature, 30 min at 4°C, 10 min on ice), followed by gap extension and religation using the Klenow fragment of DNA polymerase I in the presence of T4 DNA ligase overnight at 15°C. Recombinant constructs were transformed into competent *Escherichia coli* D1210 and appropriate mutants selected by colony hybridization using ^{32}P-labeled oligonucletide probes (32). All recombinant clones were confirmed by restriction digest analysis and double-stranded DNA sequencing.

Transcription and in vitro translation

Plasmid DNA was isolated from *E. coli* D1210 by alkaline lysis (33) and purified by CsCl density gradient ultracentrifugation as described previously (32). Five micrograms of each plasmid DNA was linearized downstream from the termination codon with Hind III and used to direct the transcription of α-tubulin mRNA in 100-μl reactions, containing 40 mM tris(hydroxymethyl)-aminomethane-HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 0.5 mM each of ATP, GTP, CTP and UTP, and 50 U of T7 RNA polymerase (Promega). Five micrograms of the resulting α-tubulin mRNAs was translated in 250-μl reactions containing 175 μl of micrococcal nuclease treated rabbit reticulocyte lysate (Promega Corp., Madison, WI), 20 μM unlabeled amino acids lacking methionine, and ~300 μCi of [^{35}S]-methionine for 60 min at 30°C. Samples were supplemented to 1.5 M glycerol, 2 mg/ml bovine serum albumin, 40 μg/ml leupeptin, and 10 μg/ml each of DNAse I and RNAse A and incubated for 15 min at 30°C. Unincorporated radiolabel and small molecular weight contaminants were then partially removed by chromatography on Sephadex G-25 columns equilibrated in PB + 2.5 M glycerol. Full-length α-tubulin was purified before coassembly using Mono-Q anion-exchange high-performance liquid chromatography (HPLC) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as previously described (32).

Taxol-based coassembly

The taxol-based coassembly assay was performed as outlined by Yaffe et al. (32). Briefly, in vitro synthesized [^{35}S] wild-type or [^{35}S] mutant α-tubulin (HPLC purified) were mixed with carrier MTP (3–8 mg/ml depending on the number of cycles of coassembly), supplemented to 1 mM GTP and induced to assemble into microtubules by incubation at 37°C for 30 min. The microtubules were then stabilized by the addition of 50 μM taxol. MAPs were displaced by bringing the samples to 0.5 M NaCl followed by incubation for an additional 20 min. The high salt wash also removes tubulin subunits that might be adventitiously bound to the microtubule. (In control studies, for example, done with native and denatured α-tubulin, 100% of the native subunit and <0.5% of the denatured subunit were observed to incorporate into microtubules using this procedure [32].) Microtubules and MAP aggregates were pelleted through two volumes of a 50% sucrose cushion (150,000 *g* for 1 h), followed by depolymerization at 4°C for 30 min in one-half the starting volume of PB + 2.5 M glycerol supplemented with 12 mM $CaCl_2$ (34). Soluble tubulin dimers were clarified of protein aggregates by centrifugation at 100,000 *g* for 20 min at 4°C, and additional cycles of assembly/disassembly were reinitiated by the addition of 15 mM

ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid, 1 mM GTP, and 20 μM taxol and performed as described above. The taxol-based coassembly is a stringent procedure that removes proteins that are not integral to the microtubule lattice. MTP used for the coassemblies consisted of ~80–85% tubulin and ~15–20% MAPs. After two cycles of assembly/disassembly, MAPs were selectively removed and the samples approached a level of purity (~100% tubulin) comparable with or in excess of that obtained by phosphocellulose chromatography (cf. reference 32).

Aliquots were removed at various stages of assembly/disassembly for scintillation counting and protein determination (30), i.e., at the start (I.S.), first microtubule pellet (P1), first depolymerized supernatant (S1) (end of first cycle), second microtubule pellet (P2), second depolymerized supernatant (S2) (end of second cycle), etc. Specific activities estimated from these measurements were plotted relative to that of the initial starting material ([^{35}S]Mono-Q purified α-tubulin in MTP carrier) taken as 100% (see Figs. 2 and 3, *A* and *B*). Specific activity of wild-type tubulin typically increases during the first cycle of coassembly/dissassembly (I.S. → P1 → S1) and remains more or less constant at a value >100% during the second and subsequent cycles of assembly/disassembly (32). This increase reflects the fact that MAPs, which constitute ~20% of the protein mass of MTP, and denatured/assembly-incompetent tubulin are selectively removed by the taxol procedure. In this study the amount of incompetent tubulin protein varied from ~5% in freshly prepared MTP (see Fig. 2) to ~25% following 2 mo of storage at −20°C (see Fig. 3), contributing to slightly different coassembly profiles. In studies with HCHO, reductively methylated tubulin dimers containing ^{35}S-labeled wild-type or mutant α-tubulin (prepared as described below) were mixed with MTP carrier (~3 mg/ml) and subjected to two cycles of taxol-based coassembly/disassembly. Specific activity at S2 measured relative to unmethylated controls were plotted as a function of HCHO concentration (see Figs. 3, *C* and *D*, and 4 *B*), and the decrease in specific activity at S2 relative to the unmethylated control was taken as a measure of coassembly inhibition (Table 1).

Temperature-dependent coassembly

In vitro synthesized wild-type or mutant [^{35}S]α-tubulin was purified on the Mono Q anion-exchange column, mixed with ca. 3 mg/ml carrier MTP in PB + 2.5 M glycerol buffer and subjected to one cycle of assembly/disassembly following Sternlicht and Ringel (28). Briefly, the mixtures were supplemented to 1 mM GTP and incubated at 37°C for 40 min. The microtubules were pelleted through 3 volumes of 50% sucrose in PB (150,000 *g*, 60–90 min). Pellets were resuspended in half the starting volume of PB + 2.5 M glycerol over 30 min at 4°C and denatured or undepolymerized protein was removed by centrifugation at 80,000 *g* for 20 min. Aliquots were removed for scintillation counting and protein determination. In contrast with the stringent taxol-based coassembly procedure that removes MAPs, this procedure retains the MAPs. In studies done with HCHO, reductively methylated tubulin dimers containing ^{35}S-labeled wild-type or mutant α-tubulin were prepared as described below, mixed with ca. 3 mg/ml MTP carrier, and subjected to one cycle of temperature-dependent coassembly. Coassembly inhibition was taken as the decrease in specific activity at S2 relative to the unmethylated control (Table 1).

Reductive methylation

Reductive methylation of MTP was performed as outlined by Szasz et al. (14). A modified procedure was used for in vitro synthesized wild-type and mutant α-tubulins. These latter samples (crudes or HPLC purified) were mixed with MTP carrier (1.5–3.0 mg/ml), supplemented with GTP to 1 mM, and incubated at 37°C for 45 min to allow exchange of the alpha-subunit into tubulin dimer. Samples were then incubated at 4°C for 45 min and tubulin dimers purified by anion-exchange HPLC (32). The purified dimers were dialyzed overnight at 4°C in PB + 2.5 M glycerol containing 0.2 mM phenylmethylsulfonyl fluoride, divided into equal aliquots, and reductively methylated using 0–4 mM HCHO in the presence of 12 mM $NaCNBH_3$ for 30 min at 37°C followed by 50 mM glycine to quench further reaction.

Kinetic analysis

Estimates of fraction unmethylated Lys 394, NH_2-termini, and bulk lysyl residues as a function of HCHO concentration (see Fig. 4) were taken from the study of Sherman et al. (17). Schiff base formation, the first step in the methylation of primary amines, is assumed to be rate limiting, whereas the reduction step is presumed to be rapid and irreversible in the presence of a sufficient excess of $NaCNBH_3$ (17, 35). If the HCHO concentration remains essentially constant during the reaction (an excellent approximation for these studies), then the mono and dimethylation reactions are pseudo-first order with overall mono- and dimethylation rate constants of k_1[HCHO] and k_2[HCHO], respectively (17). Under these conditions,

$$\text{Fraction unmethylated amino groups} = \exp(-k_1[\text{HCHO}]t), \quad (1)$$

where t = reaction time (30 min in this study). k_1 values of 0.0037, 0.011, and ~0.032 $\text{min}^{-1}\text{mM}^{-1}$ (17) for bulk lysines, NH_2-termini, and Lys 394, respectively, were used in Fig. 4.

^{3}H-tyrosylated tubulin

MTP was radiolabeled by the enzymatic addition of [^{3}H]tyrosine to the carboxy-terminus of α-tubulin as described previously (32). ^{3}H-tyrosylated tubulin was reductively methylated and either directly assembled into microtubules by the taxol-dependent procedure or mixed with carrier MTP for coassembly into microtubules.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved on 9% polyacrylamide-SDS gels following Laemmli (36). Autoradiography was performed as outlined by Bonner and Laskey (37).

RESULTS

α-Tubulin mutants

Point mutations, R390S and K394E, altering the positively charged Lys 394 region were introduced into a full-length human keratinocyte α-tubulin cDNA clone (kα1) downstream from the T7 polymerase site in pGEM3 (Materials and Methods). In R390S, a neutral serine residue was used to replace the positively charged arginine residue normally present in this position. In K394E, the highly reactive lysine residue was converted to a negatively charged glutamate. α-Tubulin mRNA was transcribed from wild-type or mutant vectors, translated in vitro in rabbit reticulocyte lysates supplemented with [^{35}S]methionine, and the translation products analyzed by SDS-PAGE following anion-exchange purification (Fig. 1). R390S and K394E comigrated with the wild-type alpha subunit produced by in vitro translation and with the α-tubulin subunit from bovine neuronal MTP.

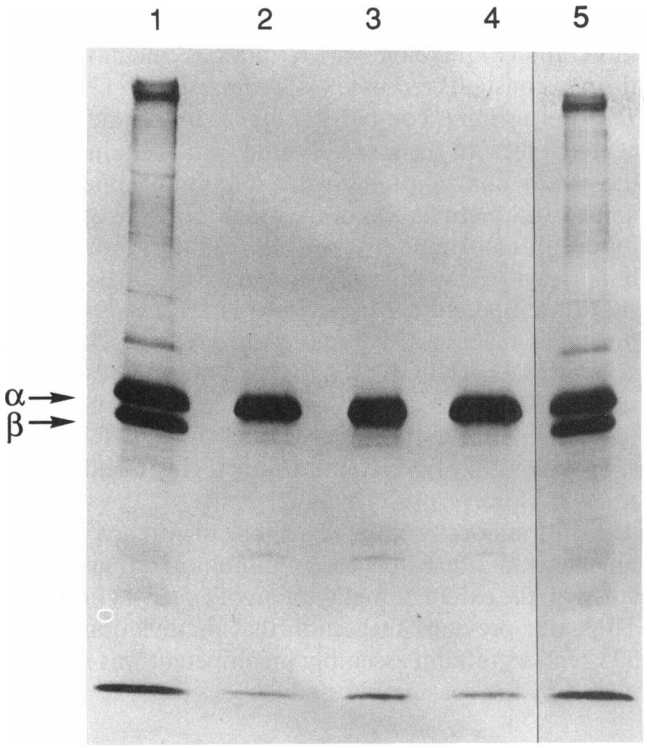

FIGURE 1 Analysis of in vitro translated α-tubulin. Aliquots containing equal ^{35}S cpm of HPLC-purified reactions (lanes *2–4*) were subjected to SDS-PAGE flourography (9% gels). Lanes *1* and *5*, ^{14}C-methylated MTP standard. Lane *2*, wild-type control. Lane *3*, R390S. Lane *4*, K394E.

Coassembly of the mutants with MTP

The functional consequences of these mutations were examined. mRNA coding for the mutations were translated in vitro in the presence of [^{35}S]methionine. The full-length translation products were purified by anion-exchange HPLC, mixed with carrier MTP, and subjected to two or more cycles of coassembly/disassembly using the stringent Taxol/NaCl/Ca^{2+} procedure (Materials and Methods). We chose this assay because it readily distinguishes proteins that are integral to the microtubule lattice, such as tubulin dimer, from proteins associated peripherally with the microtubule such as MAPs. The assay is based on taxol stabilization of microtubules followed by a dissociative treatment with high salt (0.5 M NaCl) to remove MAPs and proteins adventitiously bound to the microtubule. Previous studies have demonstrated that this procedure can accurately distinguish between specific incorporation of alpha subunits into the microtubule lattice and adventitious binding to the microtubule surface (32). As a result, this coassembly assay can quantitatively distinguish between functional and nonfunctional protein (32, 38).

As shown in Fig. 2, both the R390S and K394E mutant subunits maintained a level of specific activity through several cycles of assembly/disassembly similar to that of wild-type α-tubulin subunits. All samples showed an increase in specific activity. This increase, which is most pronounced during the first cycle of assembly/dissassembly (I.S. → P1 → S1), reflects the selective removal of MAPs (~20% by weight of MTP), as well as denatured/assembly-incompetent tubulin (estimated here as 5–10% of the MTP), and is indicative of 100% coassembly-competent tubulin (32, 38). Thus, within the limitations of the coassembly assay (see Discussion), both the R390S and K394E point mutants appear fully competent to assemble into microtubules. Taxol is known to stabilize microtubules and lower the critical tubulin concentration required for assembly. To test for the possibility that taxol might have induced the polymerization of otherwise assembly-incompetent mutant tubulin dimers, a temperature-dependent coassembly study (no taxol) of mutant K394E was also carried out (Methods). The mutant coassembled to the same extent as that of wild-type in the absence of taxol demonstrating that taxol did not induce coassembly of an incompetent form.

Mutation Glu 394 provides partial protection against reductive methylation

Previous work had suggested that low levels of reductive methylation inhibit microtubule assembly due to modification of the highly reactive lysine 394 residue in α-tubulin to its mono- and dimethylated lysine adducts (17, 32). We therefore examined whether replacement of this highly reactive lysine with a glutamate residue in the

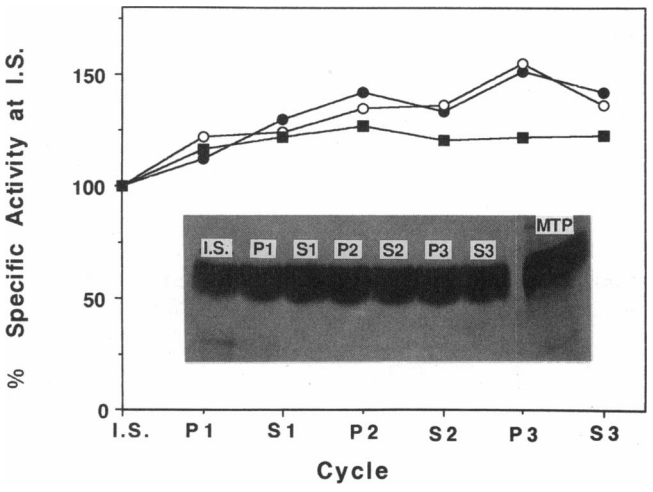

FIGURE 2 Coassembly of α-tubulin mutants. (*A*) Wild-type (○), R390S (■), and K394E (●) α-tubulins were purified on the Mono Q column, mixed with MTP carrier, and subjected to three cycles of taxol-dependent coassembly (Methods). Samples were removed at respective pellet (*P*) and supernatant (*S*) stages for specific activity determinations by scintillation counting and protein concentration measurement. The inset shows a fluorogram of an SDS-PAGE gel for K394E where equal amounts of protein from consecutive cycles were loaded. *MTP* refers to a ^{14}C-methylated MTP standard.

K394E mutant would afford protection to the assembly-inhibition observed with reductive methylation. Wild-type and mutant α-tubulin subunits, K394E or R390S, were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine, exchanged into tubulin dimer, and purified by anion-exchange HPLC. The purified dimers were reductively methylated for 30 min at 37°C with increasing concentrations of formaldehyde, mixed with carrier MTP from bovine brain, and assayed for coassembly competence by two (Fig. 3) or three (Table 1) cycles of taxol/NaCl/Ca^{2+} coassembly. Fig. 3, *A* and *C*, shows the results for K394E; Fig. 3, *B* and *D*, the results for R390S. A reductive methylation study was also carried out in the absence of taxol (Table 1, temperature-dependent assay). This study gave similar results as the taxol-based studies.

Several features are evident from these coassembly studies. (*a*) The ability of both wild-type and mutant subunits to incorporate into microtubules was progressively inhibited by increasing levels of reductive methylation (Fig. 3). In comparison with earlier assembly studies of reductively methylated dimer (assembly ~100% inhibited at ≥1 mM HCHO) (13), the inhibitory effect of reductive methylation on *coassembly* was less pronounced, even for the wild-type subunits (compare solid and dash lines in Fig. 3, *C* and *D*). The differential effects of reductive methylation on assembly versus coassembly are examined further below. (*b*) Replacement of lysine 394 by glutamic acid afforded only partial protection against the inhibitory effects of methylation. K394E coassemblies consistently displayed somewhat higher levels of specific activity than that of wild-type at all levels of methylation and at each stage of the coassembly procedure (≤30–40% greater than that of wild-type) (Fig. 3, *A* and *C*; Table 1). In contrast, no significant difference was observed between wild-type and mutant R390S (Fig. 3, *B* and *D*). (*c*) The protective effect of Glu 394 was most pronounced at low levels of formaldehyde, i.e., conditions under which we previously have shown lysine 394 to be preferentially modified relative to bulk lysines. (Thus, at 1 mM HCHO where ca. 7–9 methyls out of a possible total of ~72 were introduced into tubulin dimer and Lys 394 is known to be ~65–70% methylated [13, 17], the incorporation of wild-type and K394E into microtubules was inhibited 46 ± 5% and 30 ± 6%, respectively [Table 1]. At 4 mM HCHO, the incorporations were inhibited to essentially identical extents [~80%, Table 1].) We conclude from these observations that wild-type tubulin synthesized in reticulocyte lysate contains the highly reactive lysyl residue and that the contribution of this residue to the coassembly inhibitions is reduced in mutant K394E as expected.

We previously noted a close, essentially stoichiometric correlation between Lys 394 methylation and assembly inhibition of tubulin dimer by HCHO (13, 17). Fig. 4 *A* compares the percent assembly competence retained by tubulin dimer following reaction with HCHO with the extent of methylation of (*a*) Lys 394, (*b*) the NH_2-termini, and (*c*) the bulk lysyl residues as calculated from the rate constants reported by Sherman et al. (17). Fig. 4 *B* shows a similar comparison for wild-type and K394E coassemblies. In the assembly study with tubulin dimer, the major contribution to the inhibition appeared to come from methylation of the highly reactive lysyl residue, with contributions from the NH_2-termini and the bulk lysyl residues being minor. The coassemblies showed a different dependence on HCHO. K394E lacks the highly reactive lysyl residue and as expected was less sensitive to HCHO than native dimer. A close correlation was observed between the extent of inhibition and the predicted extent of methylation of its NH_2-termini (Fig. 4 *B*). Wild-type contained the highly reactive lysyl residue. However, its coassembly with MTP was inhibited only modestly more than that of mutant K394E. Furthermore, the extent of the inhibition did not correlate with the extent of methylation of Lys 394 (Fig. 4 *B*). Thus, our previous suggestion that methylation of Lys 394 renders tubulin assembly incompetent was not supported by the coassembly study.

Assembly versus coassembly

We were concerned that the reduced sensitivity of wild-type coassemblies to HCHO might be an artifact of the reticulocyte translation system. Coassembly studies were therefore carried out with methylated tubulin dimers derived from bovine brain. Tubulin dimers (bovine brain MTP) were radiolabeled at the COOH-terminal tyrosine residue using [^{3}H]tyrosine and tubulin tyrosine ligase (Methods). The labeled dimers were reductively methylated and assayed either for their ability to self-assemble into microtubules or diluted 12-fold with unmodified unlabeled MTP and assayed for their ability to coassemble with MTP. Low concentrations of HCHO (<1 mM) were highly effective in supressing self-assembly of tubulin dimers, thus confirming the earlier study of Szasz et al. (13), yet allowed ≥50% of these methylated dimers to coassemble into microtubules in the presence of excess unmodified tubulin (Fig. 5). Furthermore, the extent of coassembly was similar to that observed with the in vitro synthesized wild-type α-tubulin, i.e., methylation was approximately threefold more effective in inhibiting the assembly reaction of the tubulin dimer than in inhibiting its coassembly reaction. This study together with Fig. 4 argued that there were significant differences between the assembly and coassembly assays and imposed important constraints on the mechanism of assembly inhibition by reductive methylation (Discussion).

DISCUSSION

We have carried out a site-directed mutagenesis study of α-tubulin. Biochemical/biophysical characterizations of mutant proteins typically depend on in vivo expression

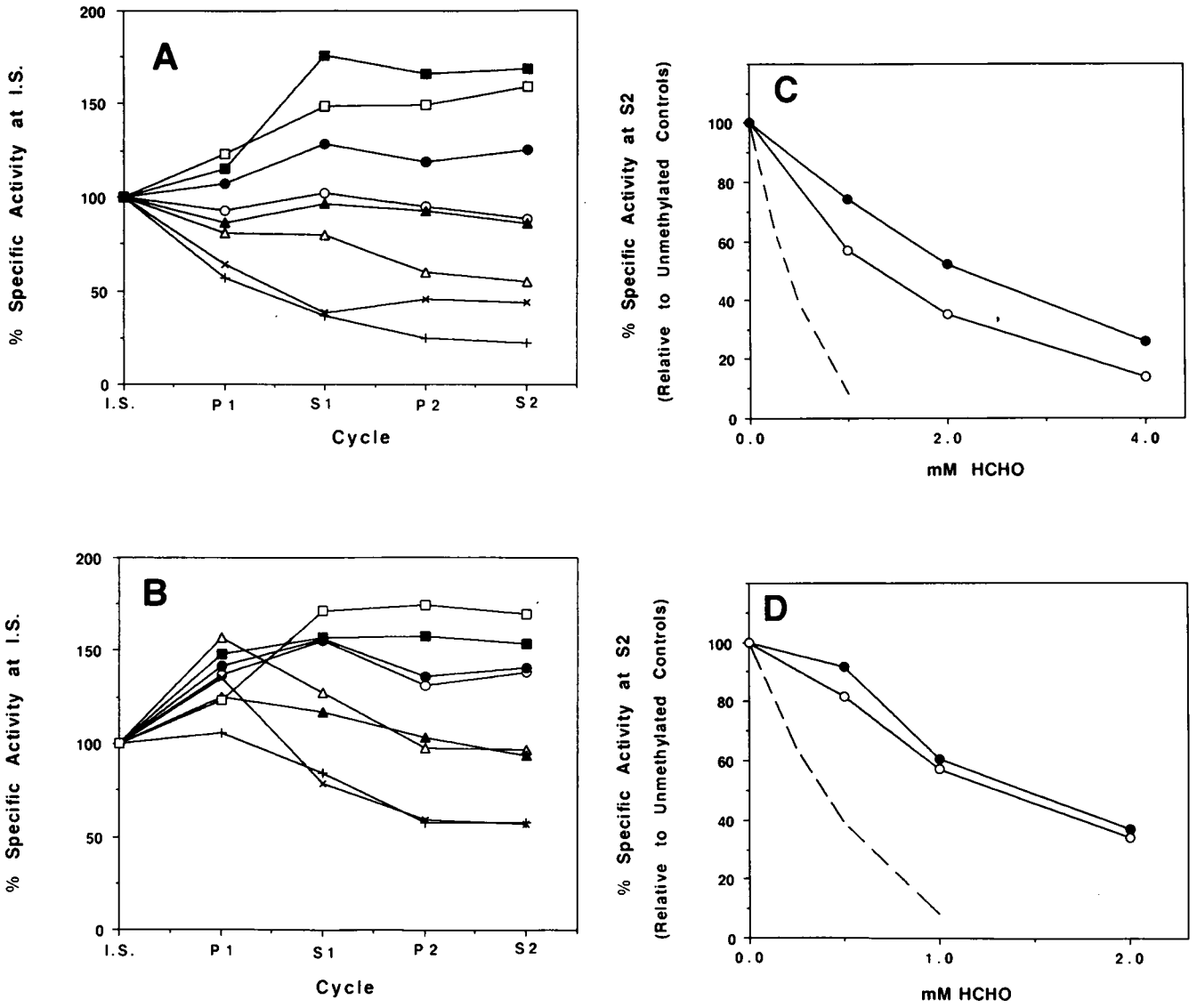

FIGURE 3 Coassembly of reductively methylated K394E and R390S mutants. α-Tubulin translation reactions (500 μl) were mixed with MTP (500 μl; ~1.5-2 mg/ml final concentration) and incubated at 37°C for 45 min to allow for exchange of the α subunits into dimer. The samples were placed on ice for ~45 min (to depolymerize microtubules formed during the incubation period) and then chromatographed on the Mono Q column to isolate radiolabeled dimers. The purified dimers were dialyzed, reductively methylated (0–4 mM HCHO, 24 mM $NaCNBH_3$) for 30 min at 37°C, mixed with carrier MTP, and subjected to two cycles of taxol-dependent coassembly as described in Fig. 2. (*A*) % Specific activity relative to I.S. measured at various stages of the coassembly for K394E mutant. (*B*) % Specific activity relative to I.S. measured at various stages of the coassembly for R390S mutant. Wild-type 0 (□); 1 (○); 2 (△); and 4 (+) mM HCHO. Mutants; 0 (■); 1 (●); 2 (▲); and 4 (×) mM HCHO. (*C* and *D*) The percent coassembly competence of wild-type (○), K394E (●), and R390S (●) as a function of HCHO concentration is plotted in *C* (K394E) and *D* (R390S). At each HCHO concentration, the % coassembly competence was taken as the % specific activity at S2 measured relative to unmethylated controls (taken from *A* and *B*). Also shown is the % assembly-competence versus HCHO concentration plot for bovine MTP (- - -) (from Szasz et al. [13], corrected for a 30-min versus a 15-min incubation with HCHO). In this study the coassemblies (no HCHO) show a larger increase in specific activity than that shown in Fig. 2, presumably due to differences in the age of the MTP preparations (see Methods).

systems to produce high levels of protein. Unfortunately, attempts to overexpress tubulin have either led to insoluble/denatured protein, as in *E. coli* (39, 40), or to lethality, as in yeast (41). In an alternative approach, we used in vitro translation in rabbit reticulocyte lysates to generate small amounts (picomoles) of highly radiolabeled mutant α subunits for investigation of the Lys 394 region of this subunit. This approach is supported by a body of literature demonstrating the utility of reticulocyte lysate systems for studying mutant proteins (cf. 42–44), as well as our studies of wild-type α- and β-tubulin and, more recently, mutant β-tubulins synthesized in reticulocyte lysate (32, 38, 45).

Lys 394 and Arg 390 are not essential for incorporation into microtubules

Alterations in the vicinity of lysine 394 resulted in the production of fully functional subunits as judged by

TABLE 1 **Coassembly competence of reductively methylated Wt and K394E Tubulin**

Coassemblies	HCHO	% Coassembly competence	
		Weight	K394E
	mM		
Taxol-based assay			
Experiment 1 (two cycles)*	0.0	100	100
	0.5	N.D.‡	N.D.
	1.0	57	74
	2.0	35	52
	4.0	14	26
Experiment 2 (three cycles)§	0.0	100	100
	0.5	64	78
	1.0	48	63
	2.0	27	36
	4.0	N.D.	N.D.
Temperature-dependent assay			
Experiment 3 (one cycle)‖	0.0	100	100
	0.5	73	88
	1.0	57	74
	2.0	48	65
	4.0	N.D.	N.D.
Average (Experiments 1–3)	0.0	100	100
	0.5	69 ± 6	83 ± 7
	1.0	54 ± 5	70 ± 6
	2.0	37 ± 10	51 ± 14

* Fig. 3 *C*. Measured at S2.
‡ Not determined.
§ Measured at S3.
‖ Measured at S1.

coassembly. Mutants K394E (net charge change: −2) and R390S (net charge change: −1) were constructed and a coassembly assay with MTP carrier protein used to assess the ability of these mutants to incorporate into microtubules (32, 38). This assay has been shown to distinguish between dimer incorporation into the microtubule lattice and nonspecific adherence to the microtubule wall and can discriminate readily against gross changes in protein conformation. K394E and R390S coassemblies were indistinguishable from wild-type (Figs. 2 and 3, no HCHO). Because the coassemblies were performed with a large molar excess of native carrier protein, we cannot completely exclude the possibility that had sufficient mutant protein been available for direct assembly studies (no added MTP), we would have found mutations K394E and R390S to be highly perturbing of assembly (cf. Fig. 5). However, we think this possibility unlikely. In a study of β-tubulin, for example, where the effects of eight point mutations on the ability of the β subunit to coassemble into microtubules was investigated, we found a broad range of coassembly behavior and were able to detect decreases in coassembly as small as 10–20% relative to wild-type (38). Furthermore, in our study of α-tubulin we have generated COOH-terminal mutants that had profound effects on coassembly (data not shown). We consequently favor the more straightforward interpretation of the coassembly data (Fig. 2), namely that residues Lys 394 and Arg 390 are not essential for the incorporation or assembly of α-tubulin into the microtubule lattice.

Reductive methylation

Although Lys 394 does not appear to be an essential residue (Fig. 2), it is nevertheless possible that its chemical modification drastically perturbs tubulin assembly.

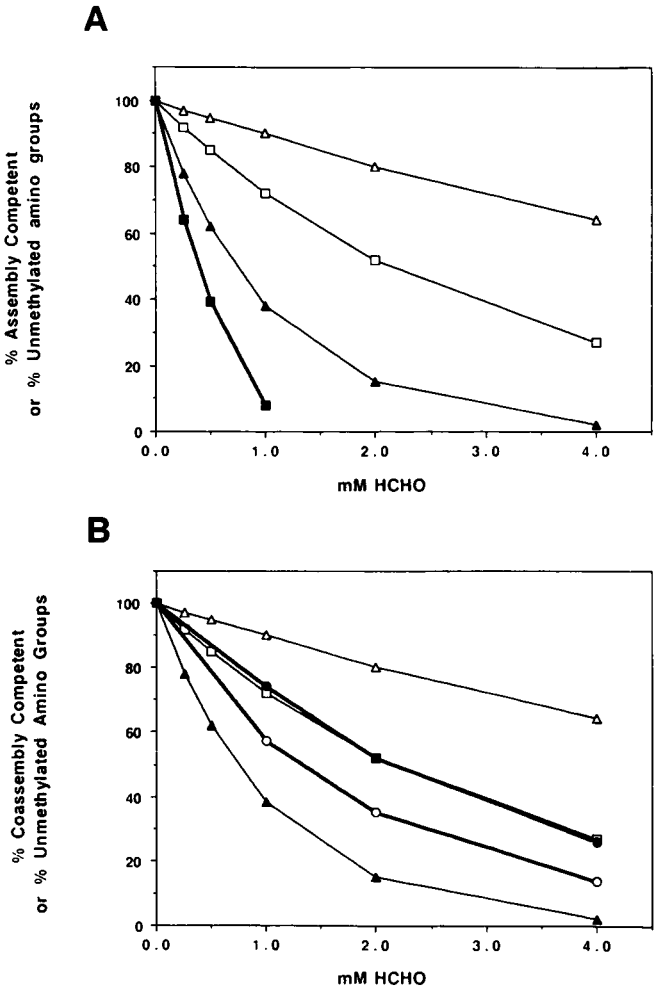

FIGURE 4 Inhibition of K394E coassembly by HCHO correlates with methylation of the NH_2-termini but inhibition of wild-type coassembly does not correlate with methylation of Lys 394. (*A*) Percent assembly competence of tubulin dimer (MTP) (■) compared with percent unmethylated Lys 394 (▲), NH_2-termini (□), and bulk lysines (△). Assembly inhibition correlates well with methylation of Lys 394 and is perceived as a superposition of contributions from methylated Lys 394 (major) and methylated NH_2-termini and bulk lysines (minor) (17). (*B*) Percent coassembly competence of wild-type (○) and K394E (●) (Table 1, average experiments 1–3) compared with percent unmethylated Lys 394 (▲), NH_2-termini (□), and bulk lysines (△). In *A* and *B* fraction unmethylated amino groups = $\exp(-k_1[\text{HCHO}]t)$, where t = 30 min and $k_1[\text{HCHO}]$ equals pseudo-first order rate constant for monomethylation (k_1 = 0.0037, 0.011, and ~0.032 $\text{min}^{-1}\text{mM}^{-1}$, respectively, for bulk lysines, NH_2-termini and Lys 394 [17]; Materials and Methods).

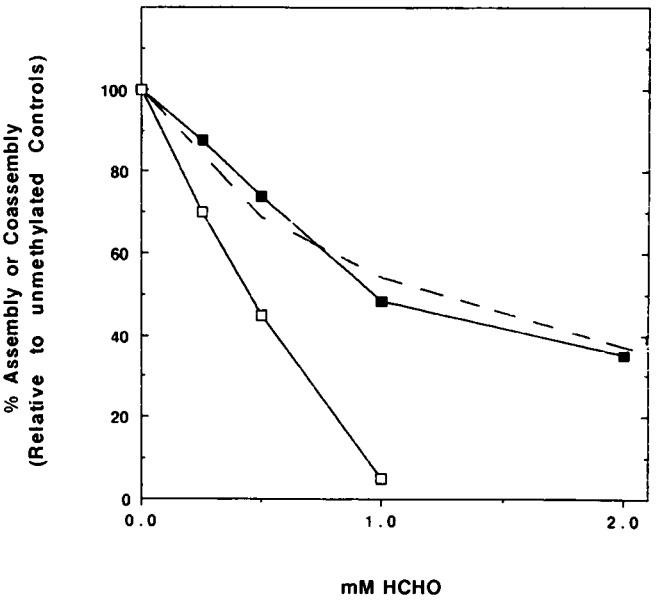

FIGURE 5 Reductive methylation of microtubule protein: assembly versus coassembly. Reductively methylated ^{3}H-tyrosylated tubulin (~1.5 mg/ml MTP) was assayed either for its ability to assemble into microtubules (□) or diluted 12-fold with unmodified unlabelled MTP (~2.5 mg/ml) and assayed for its ability to coassemble (■) through one cycle of temperature-dependent assembly/disassembly. Also shown is a plot of the % coassembly of in vitro translated wild-type α-tubulin (- - -) as a function of HCHO concentration (from Table 1, average experiments 1–3). Reductive methylation was done with 0–2 mM HCHO (30 min, 30°C) in the presence of a 12-fold molar excess of $NaCNBH_3$.

We argued previously that methylation of this highly reactive residue renders tubulin assembly incompetent. Coassembly studies of reductively methylated wild-type and K394E tubulin, however, did not support this hypothesis (Fig. 4), although these studies did suggest that methylation of this residue might account for a modest fraction (30–40%?) of the observed loss of assembly competence at low HCHO concentration (<1 mM) (Fig. 3, Table 1). It is unlikely that the reductive methylation of human keratinocyte α-tubulin (the isotype mutated in our study) is different from that of bovine α-tubulin (the wild-type tubulin isotype examined in previous studies by our laboratory) and that this difference accounts for our inability to demonstrate a significant role for K394 in reductive methylation. The α-tubulin sequence is highly conserved across species (46), and it is likely that the α-tubulin structures are also very similar. We previously tested the reactivity of a variety of α-tubulins from such diverse sources as rat, pig and bovine brain, chicken erythrocyte, rat liver, and chinese hamster ovary cells toward HCHO and have found all these isotypes contained a highly reactive Lys 394 residue (47; Blank, G., and H. Sternlicht, unpublished data). Fig. 5, which shows superimposable coassembly plots for wild-type keratinocyte and bovine tubulin after reductive methylation, also supports the notion that the reductive methylation reactions in human keratinocyte and bovine α-tubulins are similar.

Our studies implicate Lys 394 together with one or more additional residue(s) in the assembly inhibition observed with low concentrations of HCHO. One caveat to this conclusion is that the coassembly assay was carried out with small amounts of methylated protein in the presence of a large molar excess of unmodified MTP carrier protein under conditions where the effects of reductive methylation appear to be suppressed relative to studies done with high concentrations of tubulin capable of self-assembly (Figs. 4 and 5). The basis for the suppression is not understood but may reflect the fact that in the coassembly studies, the methylated subunit in the microtubule is surrounded by unmodified subunits, whereas in the assembly-studies (no added MTP), the methylated subunits are surrounded by methylated as well as unmethylated subunits. How such neighbor differences might account for the differences in sensitivity toward HCHO found for the assembly and coassembly assays is not known. One possibility is that reductive methylation affects the critical tubulin concentration for assembly. This effect would be evident in the assembly studies but not in the coassembly studies. However, our previous attempt using the Oosawa and Kasai condensation theory (48) to demonstrate that methylation affects the critical tubulin concentration failed to detect such an effect (13). Instead, our analysis suggested that methylation reduces the fraction of assembly-competent tubulin and leaves the critical concentration unaffected.

At this time, we cannot totally exclude the possibility that methylated Lys 394 functions as a potent inhibitor in the assembly assays and a much weaker inhibitor in the coassembly assays, although we favor the view that this residue plays a secondary role in both assays. It does not seem plausible to us for methylated Lys 394 to be a potent inhibitor of assembly, whereas replacement of this residue by a glutamic acid residue (Fig. 2) or its methylation (Fig. 3, *A* and *C*) has either no effect or only a modest-to-weak inhibitory effect on the ability of tubulin to incorporate into the microtubule lattice. If we accept this view, then we are confronted with several perplexing questions concerning the mechanism of assembly inhibition by HCHO and the identity of the residues contributing to the inhibition. Noting that Lys 394 is significantly more reactive toward HCHO than any other residue in tubulin, a finding that this residue is not responsible for the assembly inhibition implicates substoichiometric amounts of another as yet unidentified residue in the inhibition. Antimicrotubule drugs, such as colchicine and podophyllotoxin, form drug-tubulin complexes that coassemble with tubulin and potently inhibit microtubule assembly at substoichiometric ratios (<1:10) of complex to tubulin (28, 49–51). The basis for this process is not understood but may involve long-range distortions of the microtubule lattice. Thus, the residue(s) responsible for the inhibition need not be

much more reactive toward HCHO than the bulk lysyl residues. Rather, they need to play a functional role whose disruption by methylation causes profound effects on assembly analagous to that observed with drug-tubulin complexes. Further studies are clearly required to test this hypothesis.

Potential candidates for this residue(s) include the NH_2-termini, bulk lysyl residues, or lysyl residue(s) having intermediate reactivities (lysyl residues with reactivities intermediate between Lys 394 and the bulk lysines have not been identified but may exist in tubulin [17]). The possibility that another lysyl residue is responsible for the inhibition cannot be dismissed out of hand; however, we have no evidence linking inhibition to the methylation of a specific lysyl residue other than Lys 394. Several lines of argument strongly suggest that the NH_2-terminal methiones could be the critical residues in reductive methylation. (*a*) The NH_2-terminal methionines, which are partly buried or in a salt-bridge interaction in the native protein, are approximately one-third as reactive as Lys 394 but approximately threefold more reactive as the bulk lysyl residues (17). (*b*) The extent to which K394E coassembly is inhibited by HCHO closely parallels the extent of methylation of the NH_2-termini as calculated from the rate constants reported by Sherman et al. (17) (Fig. 4 *B*), suggesting that subunits with methylated NH_2-termini are functionally compromised. (*c*) Chemical modification and site-directed mutagenesis studies support this latter interpretation and argue that the NH_2-termini regions have an important structural role in assembly. Yaffe et al. (32), for example, showed that α-tubulin expressed in *E. coli* lysates is coassembly incompetent, whereas this subunit is fully competent when translated from α-tubulin message in rabbit reticulocyte lysates. They attributed the loss of coassembly competence to formylation of the NH_2-terminal methionine in the *E. coli* lysates. Levison et al. (24) proposed that ethoxyformylation of the NH_2-termini of tubulin by DEP causes a major loss of assembly competence at low DEP concentrations. Gu and Cowan (52) examined the ability of various amino-terminal deletions of α- and β-tubulin to coassemble into microtubles in HeLa cells and reported that removal of residues two and three has a drastic effect on the ability of the α subunit to coassemble but only a weak effect on the ability of the β subunit to coassemble. They consequently suggested that the amino-terminal region of α-tubulin may have a direct structural role in assembly.

We presently favor an inhibition model based on the NH_2-termini. We postulate the following. (*a*) Methylation of the NH_2-termini renders tubulin coassembly incompetent. That is, NH_2-terminally methylated tubulins do not incorporate readily into a microtubule lattice formed from unmodified protein (Fig. 4 *B*). (*b*) This constraint is partially relaxed during assembly where the concentration of modified tubulin is relatively high. Under these conditions, N-methylated tubulin incorporates into the lattice, probably cooperatively, and profoundly perturbs microtubule elongation, i.e., acts as a substoichiomtric poison. This proposal of a cooperative interaction between methylated tubulin dimers that overcomes their inability to coassemble with MTP has precedent in other studies of modified tubulin. Tubulins carrying deletions at the COOH-termini, for example, do not readily coassemble with unmodified tubulin (52). Nevertheless, COOH-terminally truncated tubulin dimers will readily polymerize with similarly truncated dimers, although the polymer products are abnormal (53).

At this time we do not know whether the defect responsible for the inhibition occurs in the α or β subunit, or both subunits, nor do we know what role GTP and GDP binding plays in this process. Photolabeling studies has identified residues 10–20 in β-tubulin as a potential binding site for the exchangeable GTP (54). GTP is an allosteric effector of assembly, and the finding that its binding site is close to the NH_2-terminus ultimately may prove to be significant in understanding the assembly-inhibition mechanism. It is possible that only one of the NH_2-terminii of tubulin participates in the inhibition. We previously showed that methylation of microtubule polymer does not affect tubulin activity (13). Thus, microtubule polymers can be reacted with HCHO and $NaCNBH_3$ and then depolymerized to give methylated dimers that will readily repolymerize into microtubules. This lack of effect was attributed either to a steric protection of Lys 394 and/or an alteration of its activity in the microtubule state. However, the reactivity of the NH_2-termini toward HCHO and $NaCNBH_3$ is reduced by a factor of approximately two in the microtubule polymer (17). An alternative interpretation of the microtubule protection experiment is that the NH_2-terminus of one of the subunits critical for the inhibition is inaccessible to methylating regents in the microtubule state.

Relationship to other chemical modification studies

Although our inhibition model based on the NH_2-termini is speculative, we believe it to be more satisfactory than our original model based on Lys 394. It is possible that high concentrations of Lys 394-methylated tubulin, as occurs during assembly, potently disrupts microtubule elongation, whereas low concentrations, as occurs during coassembly, allows microtubule growth. This possibility, however, is not supported by other chemical modification studies of tubulin. Smith et al. (55), for example, reported that acetaldehyde causes a substoichiometric inhibition of microtubule assembly ($\leq$0.2 mol incorporated acetaldehyde/mole tubulin inhibits assembly $\sim$100%) and proposed that modification of Lys 394 is responsible for this inhibition. If so, inhibition by acetaldehyde does not seem to require the high concentrations of modified tubulin postulated to be necessary for inhibition by HCHO. Furthermore, in the DEP study

where substoichiometric inhibition of tubulin assembly was also observed (~0.1 NH_2OH-resistant ethoxyformyl equivalents per tubulin gave ~100% inhibition), Lys 394 was not modified (24). The most straightforward interpretation of such contradictory data is that another residue other than Lys 394 is involved in substoichiometric inhibition. Interestingly, DEP and acetaldehyde are more disruptive of assembly than HCHO. Whether the differences in sensitivity simply reflect the differences in size of the modifying group, differences in the inhibition mechanism, or differences in the critical residues modified is not known at this time.

CONCLUSION

We have shown that Lys 394 of α-tubulin is not essential for incorporation of tubulin dimer into the microtubule lattice. However, the role this residue plays in the assembly inhibition induced by HCHO and $NaCNBH_3$ remains unclear. Coassembly studies suggest that Lys 394 plays a secondary role and have implicated the NH_2-terminii by default as major players in the assembly-inhibition process. The NH_2-terminal regions of the nascent tubulin chains are involved in the autoregulation of tubulin synthesis (11, 12). Cleveland and co-workers have proposed that a regulatory protein (free tubulin dimer?) binds cotranslationally to the exposed first four amino acid residues of the nascent tubulin chains (11, 12). We propose that in the native protein these residues have an additional function, i.e., have a structural role whose disturbance causes a profound, possibly substoichiometric inhibition of microtubule assembly. Additional studies will be required to test this hypothesis.

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